Original Article

The effects of bisphosphonate on pain-related behavior and immunohistochemical analyses in hindlimb-unloaded mice[☆]

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ABSTRACT

Objective: The aim of this study was to evaluate skeletal pain associated with immobility-induced osteoporosis and to examine the inhibitory effect of bisphosphonate (BP) administration on pain in hindlimb-unloaded (HU) mice.

Methods: The mechanism of osteoporotic pain in HU mice was evaluated through an examination of pain-related behavior, as well as immunohistochemical findings. In addition, the effects of alendronate (ALN), a potent osteoclast inhibitor, on these parameters were assessed.

Results: HU mice with tail suspension developed bone loss and mechanical hyperalgesia in the hindlimbs. The HU mice showed an increase in the number of calcitonin gene-related peptide (CGRP)-immunoreactive neurons and in transient receptor potential channel vanilloid subfamily member 1 (TRPV1)-immunoreactive neurons in the dorsal root ganglions (DRGs) innervating the hindlimbs. Furthermore, administration of ALN prevented HU-induced bone loss, mechanical hyperalgesia, and upregulation of CGRP and TRPV1 expressions in DRG neurons of immobility-induced osteoporotic animal models.

Conclusions: HU mice appear to be a useful model for immobility-induced osteoporotic pain and hindlimb-unloading-induced bone loss, as well as upregulation of CGRP and TRPV1 expressions in DRG neurons, and BP treatment prevented bone loss and mechanical hyperalgesia. The inhibitory effect of BP on osteoclast function might contribute to improving osteoporosis-related pain.

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1. Background

Disuse osteoporosis is a well-known result of skeletal unloading in humans during prolonged bed rest that increases the susceptibility to fractures [1]. It is commonly found in elderly patients and patients requiring prolonged bed rest or immobilization. "Locomotive syndrome", a concept proposed by the Japanese Orthopaedic Association (JOA), is a condition of reduced mobility due to impairment of locomotive organs, such as that due to knee osteoarthritis, lumbar spondylosis, and osteoporosis [2].

Osteoporosis can manifest clinically as pain, fractures, and physical disability, resulting in loss of independence and the need for long-term care. Yoshimura et al. reported that about 6.4 and 11 million individuals in Japan have lumbar spine (L2–4) and femoral neck osteoporosis, respectively [3]. A few studies found that osteoporotic hip fractures and vertebral fractures were associated with high mortality rates [4,5]. Kasai et al. reported that musculoskeletal diseases including osteoporosis were associated with a significantly higher mortality rate [6]. Therefore, osteoporosis and osteoporotic fractures are major public health problems in this aging society.

Osteoporotic pain has recently been reported as a novel form of chronic pain affecting patients with osteoporosis with no evidence of fractures who sometimes experience chronic low back pain, and this accounts for 10.4–12.5% of patients with low back pain [7,8]. In vivo studies have demonstrated marked bone loss, disruption of bone architecture, and impairment of bone mechanical properties following immobilization by tail suspension in animal models. Mice subjected to hindlimb unloading lose bone mass and have decreased bone mineralization in unloaded bones resulting from both decreased bone formation and increased bone resorption [9].

A previous report indicated that sensory innervation of ovariectomized (OVX) rat vertebrae showed increased expressions of

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calcitonin gene-related peptide (CGRP), a neuropeptide marker of pain, and transient receptor potential channel vanilloid subfamily member 1 (TRPV1), an acid-sensitive ion channel, in dorsal root ganglion (DRG) neurons [10]. The previous work of Naito et al. also showed that ovariectomy induced bone loss and mechanical hyperalgesia in hindlimbs, with upregulation of TRPV1 and CGRP expressions in DRG neurons innervating hindlimbs [11].

The objectives of the present study were to investigate pain-related behavior and to elucidate the mechanism of osteoporotic pain induced by immobility, such as bed rest after trauma, by immunohistochemical analysis of the DRG neurons in hindlimb-unloaded (HU) mice. HU mice were also treated with a bisphosphonate (BP) to elucidate the mechanisms leading to osteoporosis-related pain.

2. Materials and methods

2.1. Animals

All experiments were approved by the Animal Care Committee at Mie University and conformed to the ethics guidelines of the National Institutes of Health. A randomized, prospective, controlled, animal model design was used. Every effort was made to minimize animal suffering and the number of animals used.

Seven-week-old, male ddY mice were purchased from Japan SLC (Hamamatsu, Japan) and acclimated for 1 week before the start of the experiment. Experiments were conducted on ddY mice weighing 34–39 g, as in previous reports [9]. The mice were housed in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle (lights on from 7:00 to 19:00) and given free access to food and water. The mice were tail-suspended for 2 weeks and assigned to three groups: hindlimb-loaded mice with only tail suspension given vehicle (HL group) as the control group; HU mice with tail suspension given vehicle (HU group); and HU mice given alendronate (ALN) (HU-ALN group) (n = 8/group). Starting immediately after tail-suspension, vehicle (physiological saline) or 40 μg/kg ALN (Bonalon® Bag for I.V.; Teijin, Tokyo, Japan) was injected subcutaneously twice a week for 2 weeks, as described previously [11,12]. At the end of the 2-week tail suspension period, mechanical sensitivity of the hindlimbs was tested using von Frey filaments. Following the test, the mice were sacrificed with an intraperitoneal injection of pentobarbital sodium (0.5 mg/kg). Bilateral hindlimbs were removed to conduct micro-computed tomography (μCT), and the bilateral DRGs from L3 to L5 were collected for immunohistochemical analysis.

2.2. Hindlimb unloading

The protocol of tail suspension was modified from that of Sakai [9]. The forelimbs were normally loaded, and the movement of the hindlimbs was free without weight-bearing (unloaded). The overall suspension period was 14 days. In the hindlimb-loaded (HL) mice as the age-matched control group, the active leg was loaded via the swivel. HL mice were housed individually under the same conditions, but they were not subjected to hindlimb unloading.

2.3. Analysis of three-dimensional bone structure by μCT

After tail suspension for 2 weeks, to determine three-dimensional bone microstructure, isolated femurs and tibias were imaged using a μCT scanner (R_mCT; Rigaku Corporation, Tokyo, Japan), as described previously [11]. Three-dimensional images were reconstructed and analyzed using three-dimensional image analysis software (TRI/3D-BONE, RATOC System Engineering, Tokyo, Japan). Bone structure evaluation was performed on the

basis of bone volume fraction (BV (bone volume)/TV (tissue volume), %), trabecular number (Tb.N./mm), trabecular thickness (Tb.Th, μm), and trabecular separation (Tb.Sp, μm).

2.4. Measurement of pain-related behavior with von Frey filaments

The mechanical nociceptive threshold of the hind paw was determined, as described elsewhere [13,14]. The von Frey tests were conducted after tail suspension for 2 weeks, as described previously [11]. To evaluate the frequency of the withdrawal response, five von Frey filaments with forces of 0.4 g, 0.6 g, 1.0 g, 1.4 g, and 2.0 g were applied five times each in ascending order of force. The results are expressed as the percent response frequency of paw withdrawals. To evaluate the withdrawal threshold, each von Frey filament was applied once, starting with 0.008 g and increasing until a withdrawal response was reached, which was considered a positive response. The lowest force producing a response was considered the withdrawal threshold. To evaluate the 50% withdrawal threshold, through a wire mesh floor of the chamber, a series of nine von Frey filaments, calibrated to produce incremental forces of 0.02 g, 0.04 g, 0.07 g, 0.16 g, 0.4 g, 0.6 g, 1.0 g, 1.4 g, and 2.0 g, were applied. Data were collected using the up-down method to calculate the 50% mechanical paw withdrawal threshold [15].

2.5. Immunohistochemical analyses of DRGs

Previous studies have shown the L3–5 DRG neurons innervate the hindlimb bones [16,17]. Immunohistochemical analyses of CGRP and TRPV1 expressions were completed for the L3, L4, and L5 DRG neurons. Two weeks after tail suspension and 2 h after the behavioral tests, the mice were sacrificed, and their spines and hindlimb bones were removed. Tissues and immunostained sections were prepared as described previously [11,18]. The primary antibodies used included anti-CGRP antibody (rabbit polyclonal; Sigma-–Aldrich, St. Louis, MO, USA) or anti-VR1 C-terminus (Guinea Pig polyclonal; Neuromics, Edina, MN, USA). There were no observable immunoreactions in controls. The average percentages of immunoreactive L3, L4, and L5 DRG neuron sections were calculated on immunostained section examination. The immunostained sections were reviewed independently by three investigators.

2.6. Histological analyses of hindlimb bones

Isolated hindlimb bones and immunostained sections were prepared as described previously [18]. Sections were stained with hematoxylin and eosin (H&E) for histological analysis of bone structure. To identify osteoclasts in hindlimb bone, the tartrate-resistant acid phosphatase (TRAP) method was used. In the distal femur and the proximal tibia, the number of TRAP-positive osteoclasts was determined within an area 0.5 mm in length and 2 mm in width, apart from the most distal part of the growth plate.

2.7. Statistical analysis

Correlations among the HL group, HU group, and HU-ALN group were tested using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. A value of $p < 0.05$ was considered significant in all statistical analyses.

3. Results

3.1. Analysis of three-dimensional bone structure by μCT

The three-dimensional images of the distal femoral metaphyses (Fig. 1a) and proximal tibial metaphyses (Fig. 1b) showed less

cancellous bone in the HU group than in the HL group. Cancellous bone loss was significantly less in the HU-ALN group than in the HU group.

μCT analysis of the distal femoral metaphyses and proximal tibial metaphyses showed that BV/TV was significantly lower in the HU group than in the HL group. BV/TV was significantly higher in the HU-ALN group than in the HU group. Tb.N was significantly lower in the HU group than in the HL group, and it was significantly higher in the HU-ALN group than in the HU group. Tb.Th tended to be lower in the HU group than in the HL group, and it was significantly higher in the HU-ALN group than in the HU group. Tb.Sp was significantly higher in the HU group than in the HL group, and it was significantly lower in the HU-ALN group than in the HU group

(Supplementary data a–h can be seen by downloading the file in the Supplementary Material).

3.2. Histological analyses of hindlimb bones

The HU group had less cancellous bone in the distal femoral metaphyses and proximal tibial metaphyses than the HL group. Cancellous bone loss was less in the HU-ALN group than in the HU group (Fig. 1c). Obvious fractures of the femur and tibia were not seen on histological analysis. The numbers of TRAP-positive osteoclasts in the distal femoral metaphyses and proximal tibial metaphyses were significantly higher in the HU group than in the HL
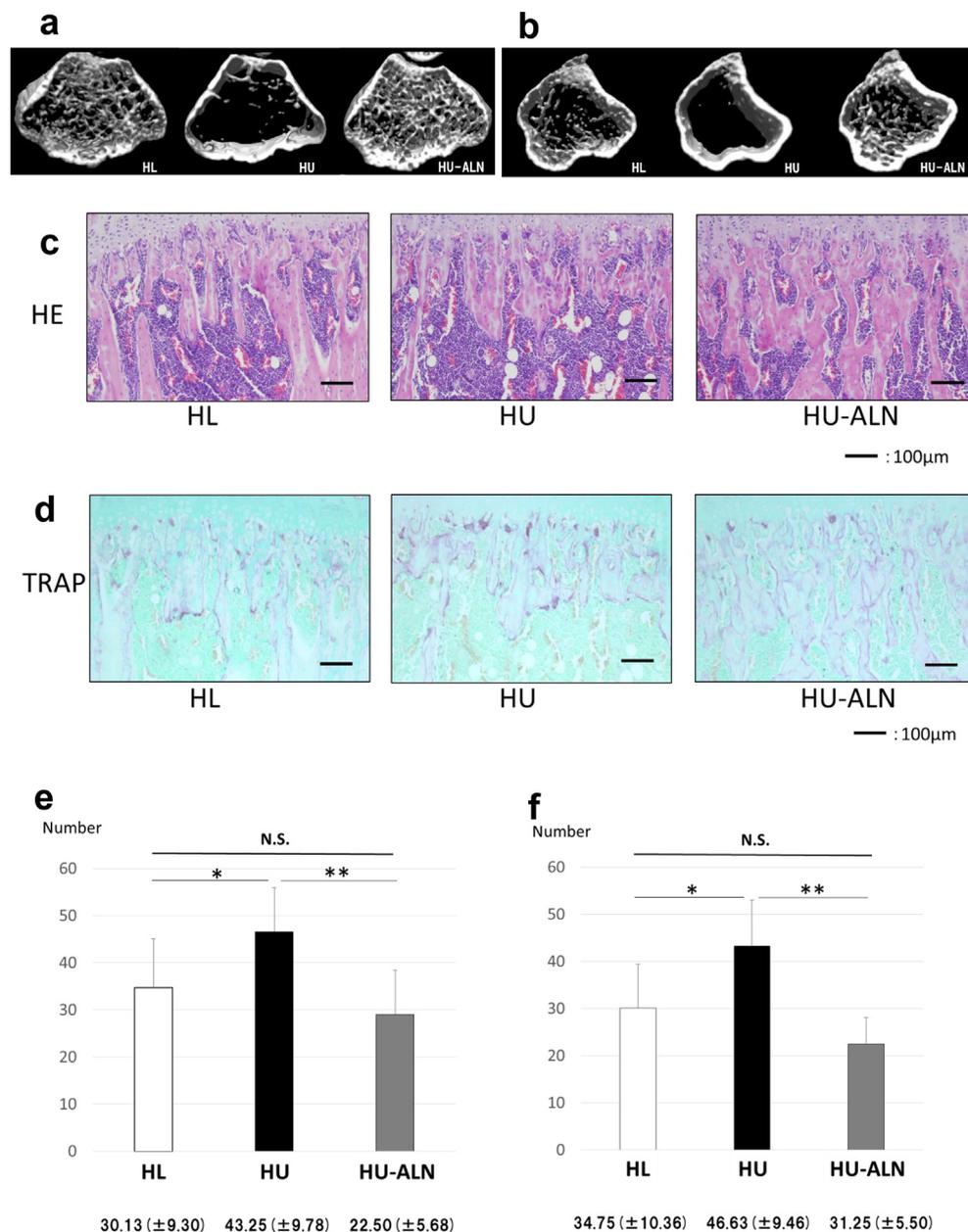
Fig. 1. μCT and histological analyses of hindlimb bone. Three-dimensional images of the distal femoral metaphysis (a) and the proximal tibial metaphysis (b). Hematoxylin and eosin (H&E) (c) and tartrate-resistant acid phosphatase (TRAP) (d) staining for histological examination of the proximal tibial metaphysis. Histological analysis of the numbers of TRAP-positive osteoclasts (mean (±SD)) in the distal femoral metaphysis (e) and the proximal tibial metaphysis (f). (*$p < 0.05$, **$p < 0.01$).

group, whereas they were significantly lower in the HU-ALN group than in the HU group (Fig. 1d–f).

3.3. Measurement of pain-related behavior with von Frey filaments

The paw withdrawal threshold was significantly lower in the HU group than in the HL group, whereas it was higher in the HU-ALN group than in the HU group (Fig. 2a). The 50% paw withdrawal threshold was also significantly lower in the HU group than in the HL group, whereas it was higher in the HU-ALN group than in the HU group (Fig. 2b). The paw withdrawal frequency stimulations (mean ($\pm$SD)) of all filaments (0.4 g, 0.6 g, 1.0 g, 1.4 g, and 2.0 g) were significantly higher in the HU group (32.5% ($\pm$12.4%), 45.0% ($\pm$15.5%), 61.25% ($\pm$11.5%), 73.75% ($\pm$9.6%), and 90% ($\pm$12.6%)) than in the HL group (10% ($\pm$16.3%), 17.5% ($\pm$16.1%), 36.25% ($\pm$15.0%), 50% ($\pm$16.3%), and 67.5% ($\pm$22.9%)), whereas they were higher in the HU-ALN group (8.75% ($\pm$12.6%), 20% ($\pm$12.6%), 32.5% ($\pm$14.4%), 47.5% ($\pm$16.1%), and 66.25% ($\pm$17.5%)) than in the HU group (Fig. 2c).

3.4. Immunohistochemical analyses of DRGs

The immunohistochemical analysis showed that the percentage of CGRP-immunoreactive L3, L4, and L5 DRG neurons was
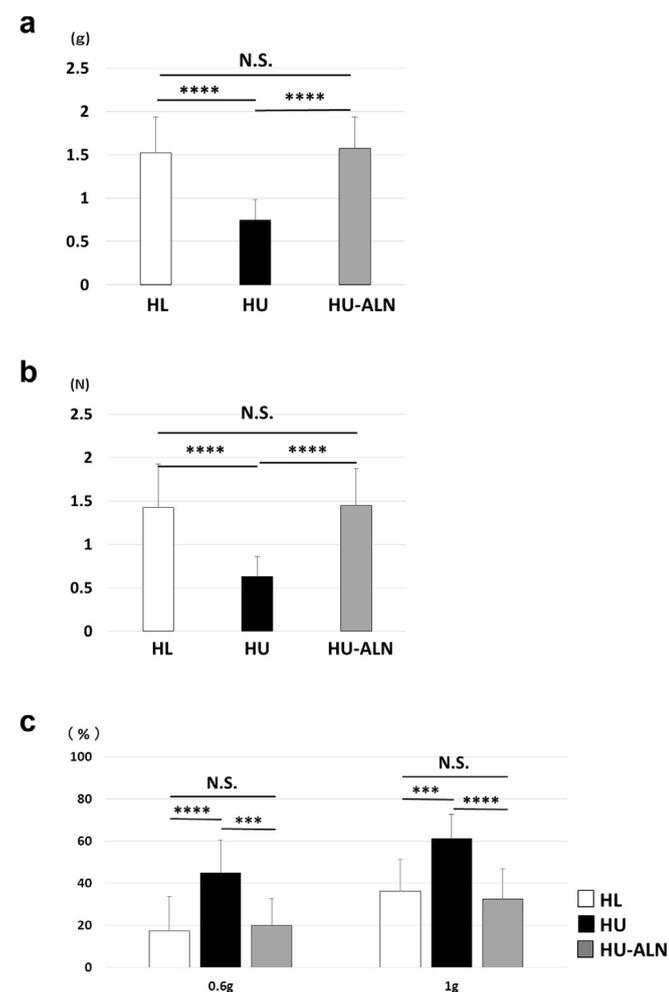
Fig. 2. Measurement of pain-related behavior with von Frey filaments. a: Paw withdrawal threshold. b: 50% paw withdrawal threshold by the up-down method. c: Withdrawal frequency stimulation. The increased paw withdrawal frequency with stimulation by all filaments (0.4–2.0 g; data shown for 0.6 g and 1.0 g filaments) in the HU group compared with the HL group is significantly decreased by ALN administration. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

significantly higher in the HU group than in the HL group, whereas it was significantly lower in the HU-ALN group than in the HU group (Fig. 3a–d).

The immunohistochemical analysis showed that the percentage of TRPV1-immunoreactive L3, L4, and L5 DRG neurons was significantly higher in the HU group than in the HL group, whereas it was significantly lower in the HU-ALN group than in the HU group (Fig. 4a–d).

4. Discussion

Mobility is critical to the well-being and quality of life of elderly patients. Immobility may be caused by a sudden event, such as bed rest after trauma. Increased bone resorption, in particular, has been shown in a recent bed rest study on bone biomarkers [19]. Thus, immobility-induced osteoporosis is well known. Osteoporosis is a skeletal disorder characterized by impaired bone strength that increases the risk of fracture. Many persons who have a vertebral fracture experience significant pain and height loss, and they may lose the ability to perform normal activities of daily living. Osteoporosis is considered a "silent disease" until a fracture occurs [20]. However, osteoporotic patients with no evidence of fractures sometimes experience vague lower back pain, which causes a significant worsening of functional capacity and deterioration in the quality of life of the people affected. Mechanisms of pain in osteoporosis are poorly known and often extrapolated based on other pathologies or other experimental models. There have been few reports regarding the mechanism. However, several studies have demonstrated that BPs, which are effective in the treatment of postmenopausal osteoporosis, improve skeletal pain in osteoporosis patients [7,8,21]. A previous study demonstrated that the acidic microenvironment created by osteoclasts during bone resorption increases bone pain in an inflammatory model [22]. The underlying mechanisms reported include activation of the acid-sensing receptors, including the TRPV1 and acid-sensing ion channels (ASICs), expressed in the sensory neurons by protons that are secreted by bone-resorbing osteoclasts. In addition, the study indicated that BP reduced the bone pain associated with increases in bone resorption [22].

In the present study, upregulation of CGRP and TRPV1 expressions was recognized in primary afferent DRG neurons innervating the hindlimbs of osteoporotic mice. CGRP has been reported to produce hyperalgesia via both protein kinase A and C second–messenger pathways. Thus, this suggests that elevated CGRP expression promotes pain [23]. TRPV1 is a ligand-gated nonselective cation channel that can be activated by capsaicin and other stimuli, such as noxious heat and low pH [24]. Hindlimb unloading leading to increased osteoclasts and bone loss may induce upregulation of CGRP and TRPV1 expressions in DRG neurons innervating the hindlimbs. In a recent clinical study, osteoporotic low back pain consisted of nociceptive pain in 85% of cases [25]. This may be the mechanism of osteoporosis-induced pain.

ALN inhibited osteoclasts and bone loss, caused an increase in the pain threshold value, and prevented CGRP and TRPV1 expressions in DRGs. The inhibitory mechanism of osteoporosis-induced pain involves increased BMD and inhibition of osteoclast activity, and osteoclast activity inhibition appears to have the stronger effect because of inhibition of TRPV1 expression in DRGs. A unique aspect of the present study is the potential correlation of CGRP and TRPV1 expressions in DRG neurons and osteoporotic pain, as well as a potential treatment strategy via BPs. In this study, it was not clear whether the sensory neurons innervating the skin, viscera, etc. showed changes in expressions of CGRP and TRPV1. The result that ALN prevented upregulation of CGRP and TRPV1 expression
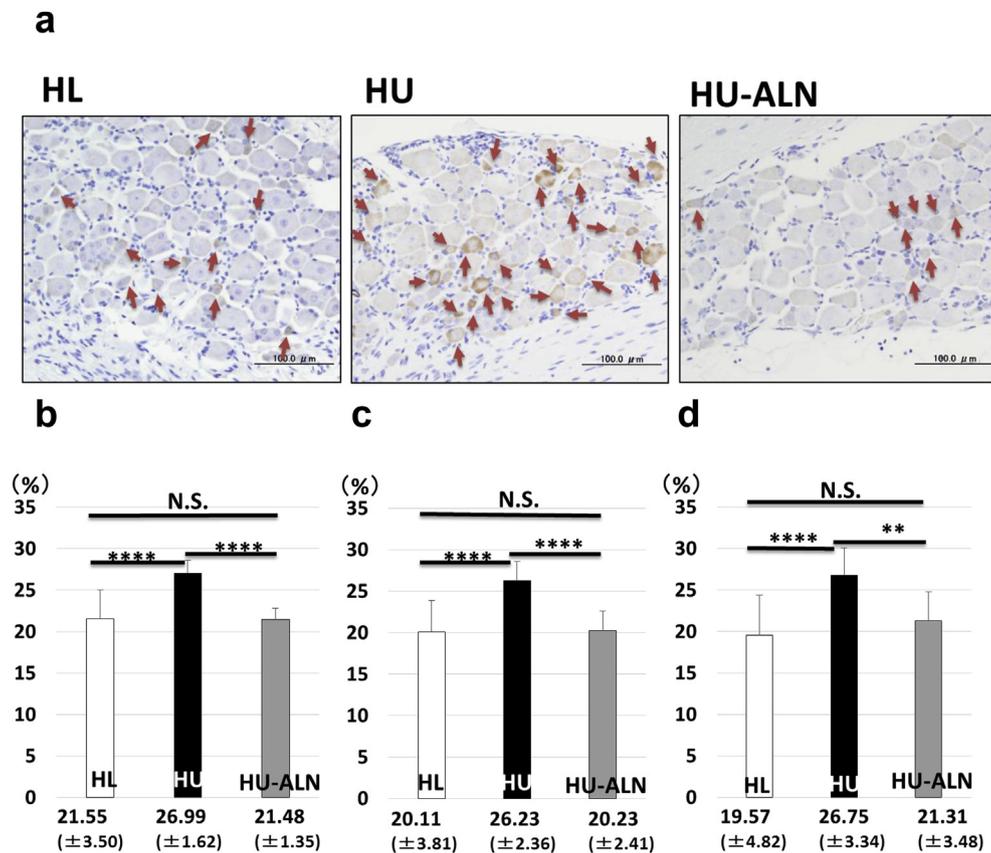
Fig. 3. Immunohistochemical analyses of CGRP expression of DRGs. a: CGRP expression in the DRG neurons, b–d: the ratios of CGRP-immunoreactive L3 (b), L4 (c), and L5 (d) DRG neurons (mean (±SD)). *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

suggests at least that the sensory neurons innervating the bone show changes in expressions of CGRP and TRPV1.

Several studies used the HU rodent model combined with BP treatment to alter bone metabolism in mice. The decreased bone mass in unloading was accompanied by increased bone resorption, as indicated by osteoclast numbers [26]. The present results indicate that the inhibitory effect of BPs on osteoclast function might contribute to an improvement in osteoporosis-related pain in immobility-induced osteoporosis patients. In clinical practice, administering ALN to immobile patients after trauma prevents osteoporosis. In addition administering ALN may be able to prevent pain and facilitate early mobilization. The duration of immobilization is the most important determinant of bone loss. Even relatively small improvements in mobility reduce the chances of osteoporosis in immobile patients.

Furthermore, in vivo models of hindlimb unloading have been previously used to study pain in rats. Chowdhury et al. reported that hindlimb suspension rats developed pressure hyperalgesia [27]. Their model evaluated the paw-withdrawal response probed with only a 15 g von Frey filament without evaluation of bone structure. In the present model, measurement of pain-related behavior with von Frey filaments was based on evaluation of the percent response frequency of paw withdrawals, the withdrawal threshold, and the 50% withdrawal threshold.

In previous reports, the tail-suspension effects on the long bones of mice were explored in both forelimb and hindlimb bones [28,29]. Material effects included lower bone density in the femora and tibiae, as well as in the humeri, of suspended mice compared to controls [28]. On the other hand, Apseloff et al. reported that tail suspension caused a significant decrease in bone density in hind

limbs, but not in forelimbs [29]. In the present study as well, forelimb bone showed decreased bone density with tail-suspension (Supplementary data I and j can be seen by downloading the file in the Supplementary Material). The three-dimensional images of the proximal humeral metaphyses showed less cancellous bone in the HU group than in the HL group. There was less cancellous bone loss in the HU-ALN group than in the HU group. However, forelimb bones were not investigated in all mice. Micro-CT analysis of the proximal humeral metaphyses showed that BV/TV tended to be lower in the HU group than in the HL group, and it tended to be higher in the HU-ALN group than in the HU group.

Clearly, prolonged bed rest and immobility may lead to loss of muscle mass and bone density. In addition, previous reports have shown that tail suspension causes significant, readily quantifiable changes in bone and muscle [29,30]. Fluckey et al. reported that HU resulted in significant reductions of bone mineral density (BMD) in cancellous regions of the distal femur and of muscle mass based on the soleus to body mass ratio. However, compared with the soleus, muscle mass or the muscle mass to body mass ratio of extensor digitorum longus (EDL) was not different between the control and HU groups [30]. Apseloff et al. reported that aminohydroxybutane bisphosphonate caused an apparent improvement in bone density in hindlimbs, but not muscle atrophy, in tail-suspended rats [29]. Thus, "osteoporosis-related pain" in the present model appears to have participated more in bone than in other tissues such as muscle.

The present study had several limitations. First, changes in neuropeptide expression in bone tissue were not directly evaluated because a neuro tracer was not used. Second, the dose-dependent effects of ALN and the effects of BPs other than ALN
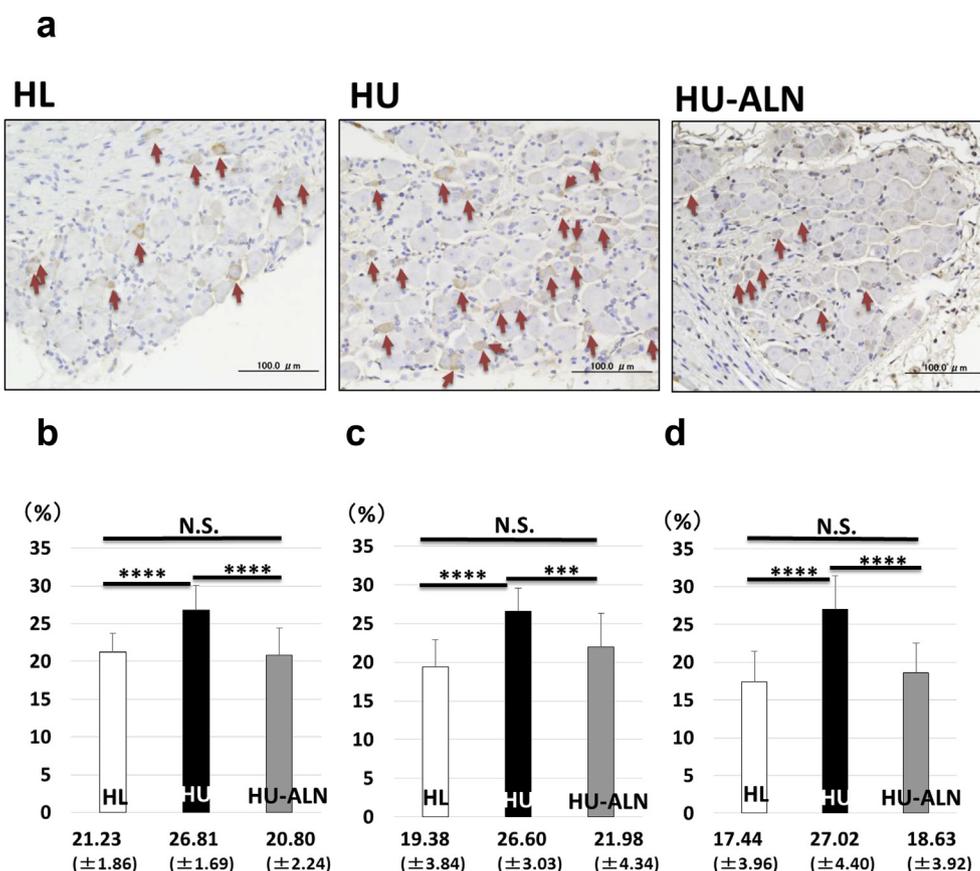
Fig. 4. Immunohistochemical analyses of TRPV1 expression of DRGs. a: TRPV1 expression in the DRG neurons, b—d: the ratios of TRPV1-immunoreactive L3 (b), L4 (c), and L5 (d) DRG neurons (mean (±SD)). *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

were not examined. Third, analysis of temporal changes in bone loss, mechanical hyperalgesia, and upregulation of CGRP and TRPV1 expressions in DRG neurons of osteoporotic mice caused by hindlimb unloading were not performed. Fourth, whether BP administration starting at the beginning prevents osteoporosis is yet to be investigated.

5. Conclusions

Hindlimb unloading mice provide a useful model of osteoporosis-related pain associated with immobility-induced osteoporosis, and ALN administration prevented osteoporosis-related pain associated with immobility-induced osteoporosis.

Competing interests

The authors declare no conflict of interests.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jos.2018.06.002.

References

[1] Watanabe Y, Ohshima H, Mizuno K, Sekiguchi C, Fukunaga M, Kohri K, Rittweger J, Felsenberg D, Matsumoto T, Nakamura T. Intravenous pamidronate prevents femoral bone loss and renal stone formation during 90-day bed rest. J Bone Miner Res 2004 Nov;19(11):1771—8.

[2] Nakamura K, Ogata T. Locomotive syndrome: definition and management. Clin Rev Bone Miner Metab 2016 May;14:56—67.

[3] Yoshimura N, Muraki S, Oka H, Mabuchi A, En-Yo Y, Yoshida M, Saika A, Yoshida H, Suzuki T, Yamamoto S, Ishibashi H, Kawaguchi H, Nakamura K, Akune T. Prevalence of knee osteoarthritis, lumbar spondylosis, and osteoporosis in Japanese men and women: the research on osteoarthritis/osteoporosis against disability study. J Bone Miner Metab 2009 Jul 1;27(5):620—8.

[4] Jacobsen SJ, Goldberg J, Miles TP, Brody JA, Stiers W, Rimm AA. Race and sex differences in mortality following fracture of the hip. Am J Publ Health 1992 Aug;82(8):1147—50.

[5] Nishimura A, Akeda K, Kato K, Asanuma K, Yamada T, Uchida A, Sudo A. Osteoporosis, vertebral fractures and mortality in a Japanese rural community. Mod Rheumatol 2014 Sep;24(5):840—3.

[6] Kasai T, Hasegawa Y, Imagama S, Sakai T, Wakai K, Suzuki K, Ishiguro N. The impact of musculoskeletal diseases on mortality-comparison with internal diseases: a 15-year longitudinal study. J Orthop Sci 2017 Nov;22(6):1126—31.

[7] Ohtori S, Akazawa T, Murata Y, Kinoshita T, Yamashita M, Nakagawa K, Inoue G, Nakamura J, Orita S, Ochiai N, Kishida S, Takaso M, Eguchi Y, Yamauchi K, Suzuki M, Aoki Y, Takahashi K. Risedronate decreases bone-resorption and improves low back pain in postmenopausal osteoporosis patients without vertebral fractures. J Clin Neurosci 2010 Feb;17(2):209—13.

[8] Terakado A, Orita S, Inage K, Kubota G, Kanzaki T, Mori H, Shinohara Y, Nakamura J, Matsuura Y, Aoki Y, Furuya T, Koda M, Ohtori S. A clinical prospective observational cohort study on the prevalence and primary diagnostic accuracy of occult vertebral fractures in aged women with acute lower back pain using magnetic resonance imaging. Pain Res Manag 2017 May 25;2017: 9265259.

[9] Sakai A, Nakamura T. Changes in trabecular bone turnover and bone marrow cell development in tail-suspended mice. J Musculoskelet Neuronal Interact 2001 Jun;1(4):387—92.

[10] Orita S, Ohtori S, Koshi T, Yamashita M, Yamauchi K, Inoue G, Suzuki M, Eguchi Y, Kamoda H, Arai G, Ishikawa T, Miyagi M, Ochiai N, Kishida S,

Takaso M, Aoki Y, Toyone T, Takahashi K. The effects of risedronate and exercise on osteoporotic lumbar rat vertebrae and their sensory innervation. Spine 2010 Oct 15;35(22):1974–82.

[11] Naito Y, Wakabayashi H, Kato S, Nakagawa T, Iino T, Sudo A. Alendronate inhibits hyperalgesia and suppresses neuropeptide mark rs of pain in a mouse model of osteoporosis. J Orthop Sci 2017 Jul;22(4):771–7.

[12] Watkins MP, Norris JY, Grimston SK, Zhang X, Phipps RJ, Ebetino FH, Civitelli R. Bisphosphonates improve trabecular bone mass and normalize cortical thickness in ovariectomized, osteoblast connexin43 deficient mice. Bone 2012 Oct;51(4):787–94.

[13] Driscoll C, Chanalaris A, Knights C, Ismail H, Sacitharan PK, Gentry C, Bevan S, Vincent TL. Nociceptive sensitizers are regulated in damaged joint tissues, including articular cartilage, when osteoarthritic mice display pain behavior. Arthritis Rheum 2016 Apr;68(4):857–67.

[14] Izumi M, Ikeuchi M, Ji Q, Tani T. Local ASIC3 modulates pain and disease progression in a rat model of osteoarthritis. J Biomed Sci 2012 Aug 21;19:77.

[15] Dixon WJ. Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 1980;20:441–62.

[16] Aso K, Ikeuchi M, Izumi M, Sugimura N, Kato T, Ushida T, Tani T. Nociceptive phenotype of dorsal root ganglia neurons innervating the subchondral bone in rat knee joints. Eur J Pain 2014 Feb;18(2):174–81.

[17] Takahashi Y, Ohtori S, Takahashi K. Sclerotomes in the thoracic and lumbar spine, pelvis, and hindlimb bones of rats. J Pain 2010 Jul;11(7):652–62.

[18] Araki K, Wakabayashi H, Shintani K, Morikawa J, Matsumine A, Kusuzaki K, Sudo A, Uchida A. Decorin suppresses bone metastasis in a breast cancer cell line. Oncology 2009 Jul 7;77(2):92–9.

[19] Armbrecht G, Belavý DL, Gast U, Bongrazio M, Touby F, Beller G, Roth HJ, Perschel FH, Rittweger J, Felsenberg D. Resistive vibration exercise attenuates bone and muscle atrophy in 56 days of bed rest: biochemical markers of bone metabolism. Osteoporos Int 2010 Apr;21(4):597–607.

[20] Gass M, Dawson-Hughes B. Preventing osteoporosis-related fractures: an overview. Am J Med 2006 Apr;119(4 Suppl 1):S3–11.

[21] Iwamoto J, Takeda T, Sato Y, Uzawa M. Effects of alendronate on metacarpal and lumbar bone mineral density, bone resorption, and chronic back pain in postmenopausal women with osteoporosis. Clin Rheumatol 2004 Oct;23(5):383–9.

[22] Nagae M, Hiraga T, Wakabayashi H, Wang L, Iwata K, Yoneda T. Osteoclasts play a part in pain due to the inflammation adjacent to bone. Bone 2006 Nov;39(5):1107–15.

[23] Sun RQ, Tu YJ, Lawand NB, Yan JY, Lin Q, Willis WD. Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization. J Neurophysiol 2004 Nov;92(5):2859–66.

[24] Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 1998 Sep;21(3):531–43.

[25] Fujimoto K, Inage K, Orita S, Yamashita M, Abe K, Yamagata M, Sainoh T, Akazawa T, Kinoshita T, Nemoto T, Hirayama J, Murata Y, Kotani T, Aoki Y, Eguchi Y, Sakuma T, Aihara T, Ishikawa T, Suseki K, Hanaoka E, Yamauchi K, Kubota G, Suzuki M, Sato J, Shiga Y, Kanamoto H, Inoue M, Kinoshita H, Koda M, Furuya T, Takahashi K, Ohtori S. The nature of osteoporotic low back pain without acute vertebral fracture: a prospective multicenter study on the analgesic effect of monthly minodronic acid hydrate. J Orthop Sci 2017 Jul;22(4):613–7.

[26] Kondo H, Ezura Y, Nakamoto T, Hayata T, Notomi T, Sorimachi H, Takeda S, Noda M. MURF1 deficiency suppresses unloading-induced effects on osteoblasts and osteoclasts to lead to bone loss. J Cell Biochem 2011 Dec;112(12):3525–30.

[27] Chowdhury P, Soulsby ME, Jayroe J, Akel NS, Gaddy D, Dobretsov M. Pressure hyperalgesia in hind limb suspended rats. Aviat Space Environ Med 2011 Oct;82(10):988–91.

[28] Simske SJ, Guerra KM, Greenberg AR, Luttges MW. The physical and mechanical effects of suspension-induced osteopenia on mouse long bones. J Biomech 1992 May;25(5):489–99.

[29] Apseloff G, Girten B, Walker M, Shepard DR, Krecic ME, Stern LS, Gerber N. Aminohydroxybutane bisphosphonate and clenbuterol prevent bone changes and retard muscle atrophy respectively in tail-suspended rats. J Pharmacol Exp Therapeut 1993 Mar;264(3):1071–8.

[30] Fluckey JD, Dupont-Versteegden EE, Montague DC, Knox M, Tesch P, Peterson CA, Gaddy-Kurten D. A rat resistance exercise regimen attenuates losses of musculoskeletal mass during hindlimb suspension. Acta Physiol Scand 2002 Dec;176(4):293–300.